Case 7670

PROTEASE CONJUGATES HAVING STERICALLY PROTECTED EPITOPE REGIONS

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CROSS REFERENCE TO RELATED APPLICATION

This application claims the benefit of U.S. Provisional Application No. 60/144,979, filed July 22, 1999.

FIELD OF THE INVENTION

The present invention relates to chemically modified subtilisin proteases which are useful in compositions such as, for example, personal care compositions, laundry compositions, hard surface cleansing compositions, and light duty cleaning compositions.

BACKGROUND OF THE INVENTION

Enzymes make up the largest class of naturally occurring proteins. One class of enzyme includes proteases which catalyze the hydrolysis of other proteins. This ability to hydrolyze proteins has typically been exploited by incorporating naturally occurring and genetically engineered proteases into cleaning compositions, particularly those relevant to laundry applications.

In the cleaning arts, the mostly widely utilized of these proteases are the serine proteases. Most of these serine proteases are produced by bacterial organisms while some are produced by other organisms, such as fungi. See Siezen et al., "Homology Modelling and Protein Engineering Strategy of Subtilases, the Family of Subtilisin-Like Serine Proteases", *Protein Engineering*, Vol. 4, No. 7, pp. 719 - 737 (1991). Unfortunately, the efficacy of the wild-type proteases in their natural environment is frequently not optimized for the artificial environment of a cleaning composition. Specifically, protease characteristics such as, for example, thermal stability, pH stability, oxidative stability, and substrate specificity are not necessarily optimized for utilization outside the natural environment of the protease.

Several approaches have been employed to alter the wild-type amino acid sequence of serine proteases with the goal of increasing the efficacy of the protease in the unnatural wash environment. These approaches include the genetic redesign and / or chemical modification of proteases to enhance thermal stability and to improve oxidation stability under quite diverse conditions.

However, because such modified proteases are foreign to mammals, they are potential antigens. As antigens, these proteases cause an immunogenic and / or allergenic response (herein collectively described as immunogenic response) in mammals.

Furthermore, while genetic redesign and chemical modification of proteases has been prominent in the continuing search for more highly effective proteases for laundry applications, such proteases have not been commercially utilized in personal care compositions and light duty detergents. A primary reason for the absence of these proteases in products such as, for example, soaps, gels, body washes, shampoos, and light duty dish detergents is due to the problem of human sensitization leading to undesirable immunogenic responses. It would therefore be highly advantageous to provide a personal care composition or light duty detergent which provides the cleansing properties of proteases without the provocation of an immunogenic response.

Presently, immunogenic response to proteases may be minimized by immobilizing, granulating, coating, or dissolving chemically modified proteases to avoid their becoming airborne. These methods, while addressing consumer exposure to airborne proteases, still present the risks associated with extended tissue contact with the finished composition and worker exposure to protease-containing dust or aerosol during manufacturing.

It has also been proposed that reduction in immunogenicity of a protease may be achieved by attaching polymers to the protease. See, e.g., U.S. Patent No. 4,179,337, Davis et al., issued December 18, 1979; U.S. Patent No. 5,856,451, Olsen et al., assigned to Novo Nordisk, issued January 5, 1999; WO 99/00489, Olsen et al., assigned to Novo Nordisk, published January 7, 1999; WO 98/30682, Olsen et al., assigned to Novo Nordisk, published July 16, 1998; and WO 98/35026, Von Der Osten et al., published August 13, 1998. However, such proposals have not suggested the importance of attaching polymers to the amino acid regions of the protease which are responsible for the immune response (*i.e.*, epitopes).

It has recently been discovered that the subtilisin protease comprises three epitope regions and that conjugation of one or more polymers, polypeptides, or other groups should be attached at one or more of these regions to effect significant reduction in immunogenicity of the protease. See, e.g., U.S. Patent Application Serial No. 09/088,912, Weisgerber et al., assigned to The Procter & Gamble Co., filed June 2, 1998.

The present inventors have discovered that steric protection near one or more of the epitope regions of the protease is an alternative mechanism to prevent or impede presentation of an epitope and decrease the immunogenicity of the protease. Accordingly, the present inventors herein provide modified subtilisins wherein the modification is at a region in steric proximity to

one or more of the epitope regions. The present inventors have therefore discovered subtilisin proteases which evoke a decreased immunogenic response yet maintain their activity as an efficient and active protease. Accordingly, the present protease conjugates are suitable for use in several types of compositions including, but not limited to, laundry, dish, hard surface, skin care, hair care, beauty care, oral care, and contact lens compositions.

SUMMARY OF THE INVENTION

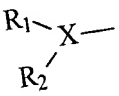
The present invention relates to protease conjugates comprising a protease moiety and one or more addition moieties, wherein each addition moiety is covalently attached to an epitope protection position of the protease moiety, wherein:

(a) the epitope protection positions for the first epitope region are selected from 1, 2, 3, 4, 5, 6, 7, 12, 17, 36, 40, 41, 43, 44, 45, 67, 86, 87, 89, 206, 209, 210, 212, 213, 214, 215, and 216 corresponding to subtilisin BPN';

(b) the epitope protection positions for the second epitope region are selected from 25, 26, 27, 46, 47, 48, 49, 50, 51, 52, 53, 54, 91, 99, 100, 101, 102, 127, 128, 129, 130, 131, 132, 133, 134, 136, 137, 138, 140, 141, 144, and 145 corresponding to subtilisin BPN'; and

(c) the epitope protection positions for the third epitope region are selected from 9, 10, 22, 23, 24, 62, 63, 143, 146, 154, 155, 156, 157, 172, 173, 187, 189, 195, 197, 203, 204, 253, 254, 256, 265, 267, 269, 271, 272, and 275 corresponding to subtilisin BPN';

and wherein the addition moieties each, independently, have the structure:



wherein X is selected from nil and a linking moiety; R_1 is selected from nil, a first polypeptide, and a first polymer; and R_2 is selected from nil, a second polypeptide, and a second polymer; wherein at least one of X, R_1, and R_2 is not nil.

The protease conjugates of the present invention have decreased immunogenicity relative to the parent protease. Accordingly, such protease conjugates are suitable for use in several types of compositions including, but not limited to, laundry, dish, hard surface, skin care, hair care, beauty care, oral care, and contact lens compositions.

DETAILED DESCRIPTION OF THE INVENTION

The essential components of the present invention are herein described below. Also included are non-limiting descriptions of various optional and preferred components useful in embodiments of the present invention.

The present invention can comprise, consist of, or consist essentially of any of the required or optional components and / or limitations described herein.

All percentages and ratios are calculated by weight unless otherwise indicated. All percentages are calculated based on the total composition unless otherwise indicated.

All component or composition levels are in reference to the active level of that component or composition, and are exclusive of impurities, for example, residual solvents or by-products, which may be present in commercially available sources.

All documents referred to herein, including all patents, patent applications, and publications, are hereby incorporated by reference in their entirety.

Referred to herein are trade names for materials including, but not limited to, enzymes. The inventors herein do not intend to be limited by materials under a certain trade name. Equivalent materials (*e.g.*, those obtained from a different source under a different name or catalog (reference) number) to those referenced by trade name may be substituted and utilized in the protease conjugates and compositions herein.

As used herein, abbreviations will be used to describe amino acids. Table I provides a list of abbreviations used herein:

Table I

Amino Acid	Three-letter Abbreviation	One-letter Abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic Acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Definitions

As used herein, the term "mutation" refers to an alteration in a gene sequence and / or an amino acid sequence produced by those gene sequences. Mutations include deletions, substitutions, and additions of amino acid residues to the wild-type protein sequence.

As used herein, the term "parent" refers to a protein (wild-type or variant) which is utilized for further modification to form a protease conjugate herein.

As used herein, the term "wild-type" refers to a protein, for example a protease or other enzyme, produced by unmutated organisms.

As used herein, the term "variant" means a protein having an amino acid sequence which differs from that of the corresponding wild-type protein.

As used herein, all polymer molecular weights are expressed as weight average molecular weights.

As referred to herein, while the conjugates of the present invention are not limited to those comprising subtilisin BPN' and variants thereof, all amino acid numbering is with reference to the amino acid sequence for subtilisin BPN' which is represented by SEQ ID NO:1. The amino acid sequence for subtilisin BPN' is further described by Wells et al., *Nucleic Acids Research*, Vol. II, pp. 7911 - 7925 (1983).

Protease Conjugates of the Present Invention

The protease conjugates of the present invention are compounds which comprise a protease moiety and one or more addition moieties, wherein the protease moiety and the addition moieties are connected *via* covalent attachment (*i.e.*, covalent bonding).

Protease Moieties

The protease moieties herein are subtilisin-like proteases, either wild-type or variants thereof. As used herein, the term "subtilisin-like protease" means a protease which has at least 50%, and preferably 80%, amino acid sequence identity with the sequences of subtilisin BPN'. Wild-type subtilisin-like proteases are produced by, for example, *Bacillus alcalophilus*, *Bacillus amyloliquefaciens*, *Bacillus amylosaccharicus*, *Bacillus licheniformis*, *Bacillus lentus*, and *Bacillus subtilis* microorganisms. A discussion relating to subtilisin-like serine proteases and their homologies may be found in Siezen et al., "Homology Modelling and Protein Engineering Strategy of Subtilases, the Family of Subtilisin-Like Serine Proteases", *Protein Engineering*, Vol. 4, No. 7, pp. 719 - 737 (1991).

Preferred protease moieties for use herein include, for example, those obtained from *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, and *Bacillus subtilis*, subtilisin BPN,

subtilisin BPN', subtilisin Carlsberg, subtilisin DY, subtilisin 309, proteinase K, and thermitase, including A/S Alcalase® (commercially available from Novo Industries, Copenhagen, Denmark), Esperase® (Novo Industries), Savinase® (Novo Industries), Maxatase® (commercially available from Genencor International Inc.), Maxacal® (Genencor International Inc.), Maxapem 15® (Genencor International Inc.), and variants of the foregoing. Especially preferred protease moieties for use herein include those obtained from *Bacillus amyloliquefaciens* and variants thereof. The most preferred protease moieties herein are subtilisin BPN' and variants thereof.

Especially preferred variants of subtilisin BPN', hereinafter referred to as "Protease A", for use as parent amino acid sequences herein are disclosed in U.S. Patent No. 5,030,378, Venegas, issued July 9, 1991, as characterized by the subtilisin BPN' amino acid sequence with the following mutations:

(a) Gly at position 166 is substituted with an amino acid residue selected from Asn, Ser, Lys, Arg, His, Gln, Ala and Glu; Gly at position 169 is substituted with Ser; and Met at position 222 is substituted with an amino acid residue selected from Gln, Phe, His, Asn, Glu, Ala and Thr; or

(b) Gly at position 160 is substituted with Ala, and Met at position 222 is substituted with Ala.

Additionally preferred variants of subtilisin BPN', hereinafter referred to as "Protease B", for use as parent amino acid sequences herein are disclosed in EP 251,446, assigned to Genencor International, Inc., published January 7, 1988, as characterized by the wild-type subtilisin BPN' amino acid sequence with mutations at one or more of the following positions: Tyr21, Thr22, Ser24, Asp36, Ala45, Ala48, Ser49, Met50, His67, Ser87, Lys94, Val95, Gly97, Ser101, Gly102, Gly103, Ile107, Gly110, Met 124, Gly127, Gly128, Pro129, Leu135, Lys170, Tyr171, Pro172, Asp197, Met199, Ser204, Lys213, Tyr214, Gly215, and Ser221; or two or more of the positions listed above combined with one or more mutations at positions selected from Asp32, Ser33, Tyr104, Ala152, Asn155, Glu156, Gly166, Gly169, Phe189, Tyr217, and Met222.

Other preferred subtilisin BPN' variants for use herein are hereinafter referred to as "Protease C", and are described in WO 95/10615, assigned to Genencor International Inc., published April 20, 1995, as characterized by the wild-type subtilisin BPN' amino acid sequence with a mutation to position Asn76, in combination with mutations in one or more other positions selected from Asp99, Ser101, Gln103, Tyr104, Ser105, Ile107, Asn109, Asn123, Leu126,

Gly127, Gly128, Leu135, Glu156, Gly166, Glu195, Asp197, Ser204, Gln206, Pro210, Ala216, Tyr217, Asn218, Met222, Ser260, Lys265, and Ala274.

Other preferred subtilisin BPN' variants for use herein, hereinafter referred to as "Protease D", are described in U.S. Patent No. 4,760,025, Estell et al., issued July 26, 1988, as characterized by the wild-type subtilisin BPN' amino acid sequence with mutations to one or more amino acid positions selected from the group consisting of Asp32, Ser33, His64, Tyr104, Asn155, Glu156, Gly166, Gly169, Phe189, Tyr217, and Met222.

The more preferred protease moieties herein are selected from the group consisting of subtilisin BPN', Protease A, Protease B, Protease C, and Protease D, with Protease D being the most preferred.

Without intending to be limited by theory, the protease moieties herein have three epitope regions: a first epitope region, a second epitope region, and a third epitope region. The first epitope region occurs at positions 70 - 84 corresponding to subtilisin BPN'; the second epitope region occurs at positions 103 - 126 corresponding to subtilisin BPN'; and the third epitope region occurs at positions 220 - 246 corresponding to subtilisin BPN'. See, e.g., U.S. Patent Application Serial No. 09/088,912, Weisgerber et al., assigned to The Procter & Gamble Co., filed June 2, 1998; copending U.S. Provisional Patent Application Serial No. 60/144,991, Rubingh et al., "Serine Protease Variants Having Amino Acid Substitutions and Deletions in Epitope Regions" filed July 22, 1999; and copending U.S. Provisional Patent Application Serial No. 60/144,980, Sikorski et al., "Serine Protease Variants Having Amino Acid Substitutions in Epitope Regions" filed July 22, 1999.

The present inventors have surprisingly discovered epitope protection positions which are in steric proximity to at least one of the foregoing epitope regions. It has further been discovered that these epitopes are protected from hydrolysis, and thus exposure of epitopes, by covalently attaching one or more addition moieties to an amino acid of the protease moiety at an epitope protection position.

The epitope protection positions which are appropriate for covalent modification with an addition moiety depend upon which epitope one desires to protect. Most preferably, at least one addition moiety is covalently attached to an epitope protection position for the first epitope region.

It has been discovered that the epitope protection positions for the first epitope region are 1, 2, 3, 4, 5, 6, 7, 12, 17, 36, 40, 41, 43, 44, 45, 67, 86, 87, 89, 206, 209, 210, 212, 213, 214, 215,

and 216 corresponding to subtilisin BPN'. Preferably, the epitope protection positions for the first epitope region are 1, 2, 3, 4, 5, 6, 7, 12, 17, 40, 41, 43, 67, 86, 87, 89, 206, 209, 214, and 215 corresponding to subtilisin BPN'. Most preferably, the epitope protection positions for the first epitope region are 1, 2, 3, 4, 5, 17, 40, 41, 43, 67, 86, 87, and 214 corresponding to subtilisin BPN'.

It has further been discovered that the epitope protection positions for the second epitope region are 25, 26, 27, 46, 47, 48, 49, 50, 51, 52, 53, 54, 91, 99, 100, 101, 102, 127, 128, 129, 130, 131, 132, 133, 134, 136, 137, 138, 140, 141, 144, and 145 corresponding to subtilisin BPN'. Preferably, the epitope protection positions for the second epitope region are 27, 47, 48, 50, 52, 102, 127, 128, 130, 131, 132, 134, 138, and 141 corresponding to subtilisin BPN'.

It has further been discovered that the epitope protection positions for the third epitope region are selected from the group consisting of 9, 10, 22, 23, 24, 62, 63, 143, 146, 154, 155, 156, 157, 172, 173, 187, 189, 195, 197, 203, 204, 253, 254, 256, 265, 267, 269, 271, 272, and 275 corresponding to subtilisin BPN'. Preferably, the epitope protection positions for the second epitope region are 22, 23, 24, 143, 146, 155, 173, 189, 197, 203, 204, 253, 254, 265, and 275 corresponding to subtilisin BPN'.

In a preferred embodiment of the present invention, the protease moiety comprises a modified sequence of a parent amino acid sequence. The parent amino acid sequence may be any of the above proteases described above, with the same preferred limitations as described above. In this embodiment, the parent amino acid sequence is substituted at one or more of the parent amino acid residues with a substituting amino acid to produce a protease moiety suitable for attachment with one or more of the present addition moieties. In accordance with the present invention, the substitution should be made at one or more of the epitope protection positions. The epitope protection positions, and preferred limitations thereof, are described above.

In order to best achieve selective attachment at one or more of the epitope protection positions of one or more addition moieties to the protease moiety, the substitution should be with a substituting amino acid which does not occur in (is unique to) the parent amino acid sequence. In this respect, any substituting amino acid which is unique to the parent amino acid sequence may be utilized. For example, because a cysteine residue does not occur in the wild-type amino acid sequence for subtilisin BPN', a substitution of subtilisin BPN' with one or more cysteine residues at one or more of the epitope protection positions is suitable for the present invention. Wherein a cysteine residue occurs at a position other than an epitope protection position of the parent amino acid sequence, it is preferable to substitute another amino acid residue for in each

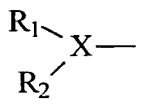
of those positions to enable selective coupling with one or more addition moieties at an epitope protection position. Cysteine is the most preferred substituting amino acid for substitution at one or more of the epitope protection positions.

Other preferred substituting amino acids include lysine. Wherein the substituting amino acid is lysine, it is preferred to mutate lysine residues which occur at positions other than an epitope protection position of the parent amino acid sequence to another amino acid residue such that functionalization of one or more of the lysine residues at an epitope protection position is selective. For example, a lysine residue occurs at position 43 of subtilisin BPN' which is an epitope protection position as defined herein. Site-selective mutation of all other lysine residues occurring in the subtilisin BPN' sequence may be performed followed by selective functionalization of the lysine residue at position 43 with an addition moiety. Alternatively, amino acid residues at any of the epitope protection positions may be mutated to lysine (for example) followed by selective functionalization at those positions by an addition moiety.

Addition Moieties

The protease conjugates of the present invention comprise one or more addition moieties wherein each of the addition moieties is covalently attached to one of the amino acid residues at an epitope protection position as described herein. The addition moiety may be any chemical structure. Preferably, the addition moiety sterically hinders the epitope protection position to which it is attached, or any other epitope protection position as defined herein. Non-limiting examples of addition moieties include organic molecules including, but not limited to, molecules having a molecular weight of less than about 1600, preferably less than about 800, more preferably less than about 400, and most preferably less than about 300; polypeptides; and polymers. As used herein, the term "polypeptide" means a molecule comprising two or more amino acid residues. As used herein, the term "polymer" means any molecule which comprises two or more identical (preferably five or more identical) monomer units.

Preferably, the addition moiety has the structure:



wherein X is selected from nil and a linking moiety; R_1 is selected from the group consisting of nil, a first polypeptide, and a first polymer; and R_2 is selected from the group consisting of nil, a second polypeptide, and a second polymer, wherein at least one of X, R_1, and R_2 is not nil.

Preferably, the protease conjugate comprises from 1 to about 15, more preferably from about 2 to about 10, and most preferably from about 1 to about 5 addition moieties.

Wherein R_1 and R_2 are each, independently, polypeptide moieties or polymer moieties, R_1 and R_2 may be identical or different. Preferably, wherein R_1 is a polypeptide moiety, R_2 is selected from nil and a polypeptide moiety, and is most preferably nil. Most preferably, wherein R_1 is a polypeptide moiety, R_2 is selected from nil and an identical polypeptide moiety, and is most preferably nil. Preferably, wherein R_1 is a polymer moiety, R_2 is selected from nil and a polymer moiety. Most preferably, wherein R_1 is a polymer moiety, R_2 is selected from nil and an identical polymer moiety. Wherein at least one of R_1 and R_2 are respectively, the first polymer and the second polymer, then X is preferably not nil.

Polypeptide Moieties

The polypeptide moieties described herein include those comprising two or more amino acid residues. Preferred polypeptide moieties are selected from proteins, including enzymes. Preferred enzymes include proteases, cellulases, lipases, amylases, peroxidases, microperoxidases, hemicellulases, xylanases, phospholipases, esterases, cutinases, pectinases, keratinases, reductases (including, for example, NADH reductase), oxidases, phenoloxidases, lipoxygenases, ligninases, pullulanases, tannases, pentosanases, malanases, β-glucanases, arabinosidases, hyaluronidase, chondroitinase, laccases, transferases, isomerases (including, for example, glucose isomerase and xylose isomerase), lyases, ligases, synthetases, and fruit-based enzymes (including, for example, papain). More preferred enzymes for use as polypeptide moieties include proteases, cellulases, amylases, lipases, and fruit-based enzymes, with proteases being even more preferred.

Examples of lipases for use as a polypeptide moiety include those derived from the following microorganisms: *Humicola*, *Pseudonomas*, *Fusarium*, *Mucor*, *Chromobacterium*, *Aspergillus*, *Candida*, *Geotricum*, *Penicillium*, *Rhizopus*, and *Bacillus*.

Examples of commercial lipases include Lipolase®, Lipolase Ultra®, Lipozyme®, Palatase®, Novozym435®, and Lecitase® (all of which are commercially available from Novo Nordisk, Copenhagen, Denmark), Lumafast® (commercially available from Genencor, Int., Rochester, NY), and Lipomax® (Genencor, Int.).

Examples of proteases for use as the polypeptide moiety include serine proteases, chymotrypsin, and elastase-type enzymes. The most preferred proteases for use as a polypeptide moiety include serine proteases, as were defined herein above in the discussion of "protease moieties".

Most preferably, wherein the polypeptide moiety is a serine protease, the polypeptide moiety carries, independently, the definition of a protease moiety as described herein above. Preferably, as described above, the polypeptide moiety has a modified amino acid sequence of a parent amino acid sequence wherein the modification is in one or more of the epitope protection positions as described herein above (which parent amino acid sequence may be referred to as a "second" parent amino acid sequence). In this instance, one of the linking moiety (wherein the linking moiety is not nil) or the protease moiety (wherein the linking moiety is nil) is covalently attached to the polypeptide moiety through one of the substituting amino acids present in one of the epitope protection positions of the polypeptide moiety. Wherein the polypeptide moiety is a serine protease, the same preferred groupings of epitope protection positions apply as are described herein above for protease moieties and their corresponding parent amino acid sequences.

Most preferably, wherein the polypeptide moiety is a serine protease, the polypeptide moiety and the protease moiety are equivalent moieties. In this instance, the polypeptide moiety and the protease moiety are most preferably attached through a disulfide bridge, wherein X is nil, and most preferably, R_2 is nil.

Polymer Moieties

The addition moieties herein may comprise a polymer moiety. As used herein, the term polymer moiety means any molecule which comprises two or more identical (preferably five or more identical) monomer units. Examples of suitable polymer moieties include polyalkylene oxides, polyalcohols, polyvinyl alcohols, polycarboxylates, polyvinylpyrrolidones, celluloses, dextrans, starches, glycogen, agaroses, guar gum, pullulan, inulin, xanthan gum, carrageenan, pectin, alginic acid hydrosylates, and hydrosylates of chitosan. Preferred polyalkylene oxides include polyethylene glycols, methoxypolyethylene glycols, and polypropylene glycols. Preferred dextrans include carboxymethyldextrans. Preferred celluloses include methylcellulose, carboxymethylcellulose, ethylcellulose, hydroxyethyl cellulose, carboxyethyl cellulose, and hydroxypropylcellulose. Preferred starches include hydroxyethyl starches and hydroxypropyl starches. The more preferred polymers are polyalkylene oxides. The most preferred polymer moiety is polyethylene glycol.

Wherein R_1 and R_2 are each, independently, polymer moieties, R_1 and R_2 preferably has a collective molecular weight (*i.e.*, molecular weight of R_1 plus molecular weight of R_2) of from about 0.2 kD (kilodaltons) to about 40kD, more preferably from about 0.5 kD to about 40 kD,

even more preferably from about 0.5 kD to about 20 kD, and most preferably from about 1 kD to about 10 kD.

Wherein R_1 and R_2 are each polymer moieties, R_1 and R_2 each, independently, preferably have a molecular weight of about 0.1 kD to about 20kD, more preferably from about 0.25 kD to about 20 kD, even more preferably from about 0.5 kD to about 10 kD, and most preferably from about 0.5 kD to about 5 kD.

Wherein R_1 and R_2 are each polymer moieties, the ratio of the molecular weights of R_1 to R_2 preferably ranges from about 1:10 to about 10:1, more preferably from about 1:5 to about 5:1, and most preferably from about 1:3 to about 3:1.

Wherein R_1 is a polymer moiety and R_2 is nil, R_1 preferably has a molecular weight of from about 0.1 kD to about 40kD, more preferably about 0.5 kD to about 40 kD, even more preferably from about 0.5 kD to about 20 kD, and most preferably from about 1 kD to about 10 kD.

Linking Moieties

As used herein, X may be nil or a linking moiety which is optionally covalently attached to one or more polypeptide moieties or one or more polymer moieties, or both, and is also covalently attached to an amino acid residue at one of the epitope protection positions of the protease moiety. The linking moiety may be, generally, any small molecule, *i.e.*, a molecule having a molecular weight of less than about 1600, preferably less than about 800, more preferably less than about 400, and most preferably less than about 300. The most preferred linking moieties include those capable of being covalently bound to a cysteine residue or a lysine residue, most preferably a cysteine residue.

Examples of linking moieties and related chemistry are disclosed in U.S. Patent No. 5,446,090, Harris, issued August 29, 1995; U.S. Patent No. 5,171,264, Merrill, issued December 15, 1992; U.S. Patent No. 5,162,430, Rhee et al., issued November 10, 1992; U.S. Patent No. 5,153,265, Shadle et al., issued October 6, 1992; U.S. Patent No. 5,122,614, Zalipsky, issued June 16, 1992; Goodson et al., "Site-Directed Pegylation of Recombinant Interleukin-2 at its Glycosylation Site", *Biotechnology*, Vol. 8, No. 4, pp. 343 - 346 (1990); Kogan, "The Synthesis of Substituted Methoxy-Poly(ethylene glycol) Derivatives Suitable for Selective Protein Modification", *Synthetic Communications*, Vol. 22, pp. 2417 - 2424 (1992); and Ishii et al., "Effects of the State of the Succinimido-Ring on the Fluorescence and Structural Properties of Pyrene Maleimide-Labeled αα-Tropomyosin", *Biophysical Journal*, Vol. 50, pp. 75 - 80 (1986).

The most preferred linking moiety is substituted (for example, alkyl) or unsubstituted succinimide.

As further examples, the following non-limiting reagents may be utilized to form the linking moiety: N-[alpha-maleimidoacetoxy]succinimide ester; N-5-azido-2-nitrobenzoyloxysuccinimide; bismaleimidohexane; N-[beta-maleimidopropyloxy]succinimide ester; bis[2-(succinimidyloxycarbonyloxy)-ethyl]sulfone; bis[sulfosuccinimidyl]suberate; 1,5-difluoro-2,4-dintrobenzene; dimethlyadipimate • 2 HCl; dimethylpimelimidate • 2 HCl; dimethylsuberimidate • 2 HCl; disuccinimidyl glutarate; disuccinimidyl suberate; *m*-maleimidobenzoyl-N-hydroxysuccinimide ester; N-hydroxysuccinimidyl-4-azidosalicylic acid; N-succinimidyl-6-[4'-azido-2'-nitrophenylamino]hexanoate; N-hydroxysuccinimidyl 2,3-dibromopropionate; succinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate; succinimidyl 4-(*p*-maleimidophenyl)-butyrate; succinimidyl-6-[(beta-maleimidopropionamido)hexanoate]; bis[2-(sulfosuccinimidyloxycarbonyloxy)-ethyl]sulfone; N-[gamma-maleimidobutyryloxy]sulfosuccinimide ester; N-hydroxysulfosuccinimidyl-4-azidobenzoate; N-[kappa-maleimidoundecanoyloxy]sulfosuccinimide ester; *m*-maleimidobenzoyl-N-hydroxysulfosuccinimide ester; sulfosuccinimidyl[4-azidosalicylamido]hexanoate; sulfosuccinimidyl 7-azido-4-methylcoumarin-3-acetate; sulfosuccinimidyl 6-[4'-azido-2'-nitrophenylamino]hexanoate; sulfosuccinimidyl 4-[*p*-azidophenyl]butyrate; sulfosuccinimidyl[4-iodoacetyl]aminobenzoate; sulfosuccinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate; and sulfosuccinimidyl 4-(p-maleimidophenyl)-butyrate. Each of these reagents is commercially available from Pierce Chemical Co., Rockford, IL.

Optional Moieties

The protease conjugate may additionally comprise one or more other chemical structures, including (for example) one or more small molecules, polypeptides, and / or polymers attached to other residues of the protease not herein exemplified or even at an epitope protection position not bearing an addition moiety (herein referred to as "supplementary moieties"). Supplementary moieties may include polypeptide moieties, polymer moieties, and linking moieties as described herein above. Additionally, for example, one or more polymers (most preferably polyethylene glycol) having a molecular weight of from about 100 Da to about 5000 Da, preferably from about 100 Da to about 2000 Da, more preferably from about 100 Da to about 1000 Da, still more preferably from about 100 Da to about 750 Da, and most preferably about 300 Da may be covalently attached to the protease moiety herein at residues other than those exemplified herein.

Such polymer moieties may be attached directly to the protease moiety herein, at any location of the protease moiety, using techniques as described herein and as well-known in the art (including through a linking moiety as described herein). Non-limiting examples of polymer conjugation of this optional type is set forth in WO 99/00849, Olsen et al., Novo Nordisk A/S, published January 7, 1999.

Method of Making

The protease moieties having a substitution in one or more of the epitope protection positions (or any other location of the moiety) are prepared by mutating the nucleotide sequences that code for a parent amino acid sequence. Such methods are well-known in the art; a non-limiting example of one such method is set forth below:

A phagemid (pSS-5) containing the wild-type subtilisin BPN' gene (Mitchison, C. and J.A. Wells, "Protein Engineering of Disulfide Bonds in Subtilisin BPN'", *Biochemistry*, Vol. 28, pp. 4807 - 4815 (1989) is transformed into *Escherichia coli dut- ung-* strain CJ236 and a single stranded uracil-containing DNA template is produced using the VCSM13 helper phage (Kunkel et al., "Rapid and Efficient Site-Specific Mutagenesis Without Phenotypic Selection", *Methods in Enzymology*, Vol 154, pp. 367 - 382 (1987), as modified by Yuckenberg et al., "Site-Directed *in vitro* Mutagenesis Using Uracil-Containing DNA and Phagemid Vectors", Directed Mutagenesis - A Practical Approach, McPherson, M. J. ed., pp. 27 - 48 (1991). Primer site-directed mutagenesis modified from the method disclosed in Zoller, M. J., and M. Smith, "Oligonucleotide - Directed Mutagenesis Using M13 - Derived Vectors: An Efficient and General Procedure for the Production of Point Mutations in any Fragment of DNA", *Nucleic Acids Research*, Vol. 10, pp. 6487 - 6500 (1982) is used to produce all mutants (essentially as presented by Yuckenberg et al., *supra*).

Oligonucleotides are made using a 380B DNA synthesizer (Applied Biosystems Inc.). Mutagenesis reaction products are transformed into *Escherichia coli* strain MM294 (American Type Culture Collection *E. coli* 33625). All mutations are confirmed by DNA sequencing and the isolated DNA is transformed into the *Bacillus subtilis* expression strain PG632 (Saunders et al., "Optimization of the Signal-Sequence Cleavage Site for Secretion from *Bacillus subtilis* of a 34-Amino Acid Fragment of Human Parathyroid Hormone", *Gene*, Vol. 102, pp. 277 - 282 (1991) and Yang et al., "Cloning of the Neutral Protease Gene of *Bacillus subtilis* and the Use of the Cloned Gene to Create an *in vitro* - Derived Deletion Mutation", *Journal of Bacteriology*, Vol. 160, pp. 15 - 21 (1984).

Fermentation is as follows. *Bacillus subtilis* cells (PG632) containing the protease of interest are grown to mid-log phase in one liter of LB broth containing 10 g/L glucose, and inoculated into a Biostat C fermentor (Braun Biotech, Inc., Allentown, PA) in a total volume of 9 liters. The fermentation medium contains yeast extract, casein hydrosylate, soluble - partially hydrolyzed starch (Maltrin M-250), antifoam, buffers, and trace minerals (see "Biology of Bacilli: Applications to Industry", Doi, R. H. and M. McGloughlin, eds. (1992)). The broth is kept at a constant pH of 7.5 during the fermentation run. Kanamycin (50 μg/mL) is added for antibiotic selection of the mutagenized plasmid. The cells are grown for 18 hours at 37 °C to an A_{600} of about 60 and the product harvested.

The fermentation broth is taken through the following steps to obtain pure protease. The broth is cleared of *Bacillus subtilis* cells by tangential flow against a 0.16 μm membrane. The cell-free broth is then concentrated by ultrafiltration with a 8,000 molecular weight cut-off membrane. The pH is adjusted to 5.5 with concentrated MES buffer (2-(N-morpholino)ethanesulfonic acid). The protease is further purified by cation exchange chromatography with S-sepharose and elution with NaCl gradients. See Scopes, R. K., "Protein Purification Principles and Practice", Springer-Verlag, New York (1984)

A *p*NA assay (DelMar et al., *Analytical Biochemistry*, Vol. 99, pp. 316 - 320 (1979)) is used to determine the active protease concentration for fractions collected during gradient elution. This assay measures the rate at which *p*-nitroaniline is released as the protease hydrolyzes the soluble synthetic substrate, succinyl-alanine-alanine-proline-phenylalanine-*p*-nitroaniline (sAAPF-*p*NA). The rate of production of yellow color from the hydrolysis reaction is measured at 410 nm on a spectrophotometer and is proportional to the active protease moiety concentration. In addition, absorbance measurements at 280 nm are used to determine the total protein concentration. The active protease/total-protein ratio gives the protease purity, and is used to identify fractions to be pooled for the stock solution.

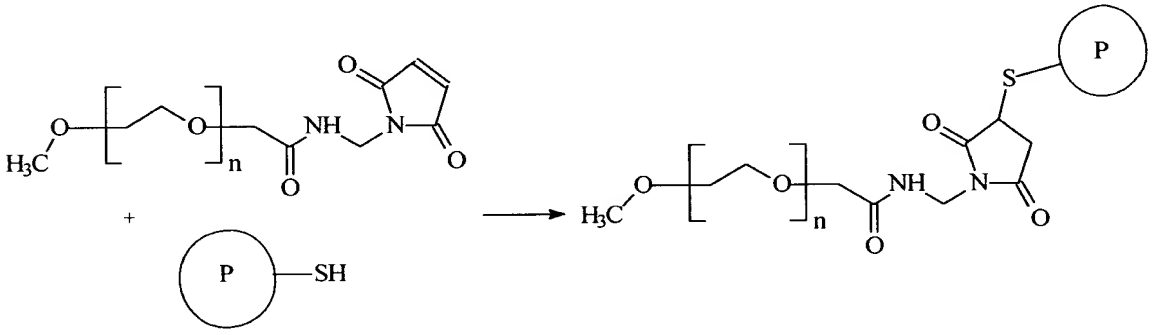
To avoid autolysis of the protease during storage, an equal weight of propylene glycol is added to the pooled fractions obtained from the chromatography column. Upon completion of the purification procedure the purity of the stock protease solution is checked with SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and the absolute enzyme concentration is determined *via* an active site titration method using trypsin inhibitor type II-T: turkey egg white (Sigma Chemical Company, St. Louis, Missouri).

In preparation for use, the protease stock solution is eluted through a Sephadex-G25 (Pharmacia, Piscataway, New Jersey) size exclusion column to remove the propylene glycol and

exchange the buffer. The MES buffer in the enzyme stock solution is exchanged for 0.01 M KH_2PO_4 solution, pH 5.5.

With the protease prepared it may be utilized for functionalization with one or more addition moieties to produce the protease conjugate. The precursor to the addition moiety (the precursor to the addition moiety reacts with the precursor to the protease moiety to form the protease conjugate which is comprised of the addition moiety and the protease moiety) is preferably activated to enhance reactivity with the precursor to the protease moiety. Such activation is well-known in the art. Non-limiting examples of methods of protease conjugate preparation are provided below.

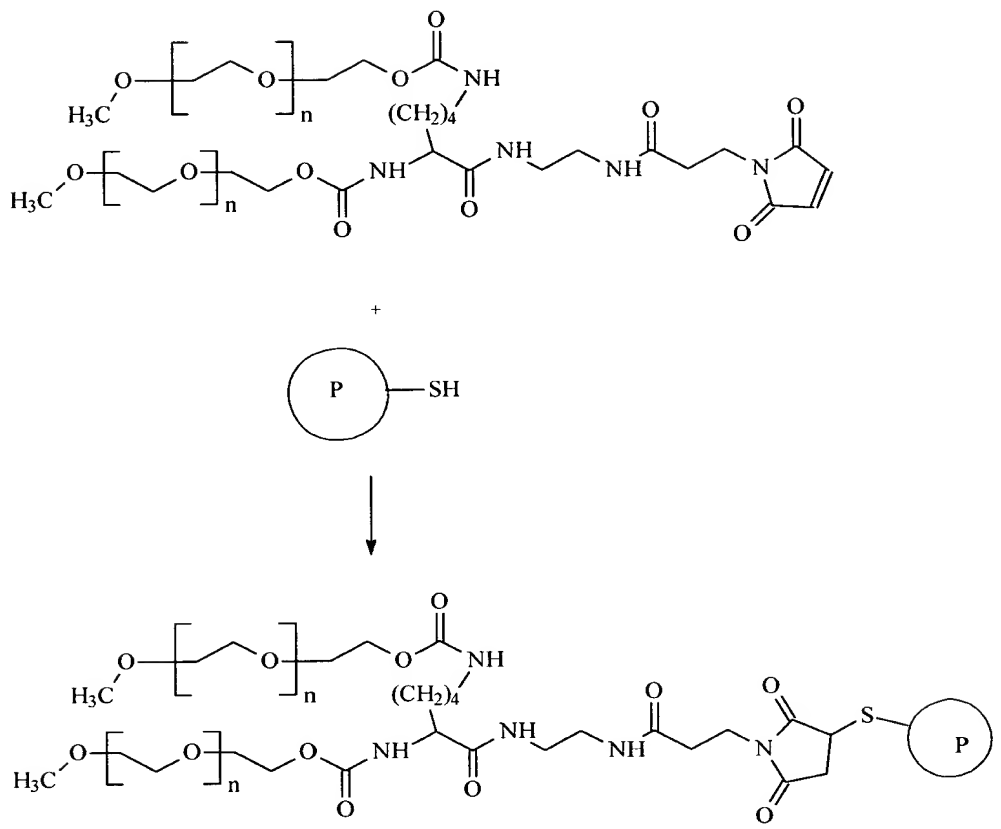
Example 1



A protease comprising a cysteine residue at one of the epitope protection positions is coupled with a polymer moiety according to the above scheme using the following method (wherein "P" represents the protease moiety minus the thiol group resulting from the cysteine substitution and n is the number of repeating monomer units of the polyethylene glycol (for example, n = 77).

A variant of subtilisin BPN' with a substitution of leucine for tyrosine at position 217 and a substitution of cysteine for serine at position 3 is prepared. A concentration of approximately 2 mg / mL in phosphate buffer (pH 5.5) of the variant is achieved. The pH is then raised to 7.5 with dilute sodium hydroxide. The variant is mixed with the monomethyl polyethylene glycol maleimide at a 25:1 activated polymer to variant excess. After one hour of mixing at ambient temperature, the pH of the mixture is adjusted to 5.5 with dilute phosphoric acid and filtered through a molecular weight cut-off ultrafilter to remove excess polymer. The concentrate contains the purified protease conjugate.

Example 2

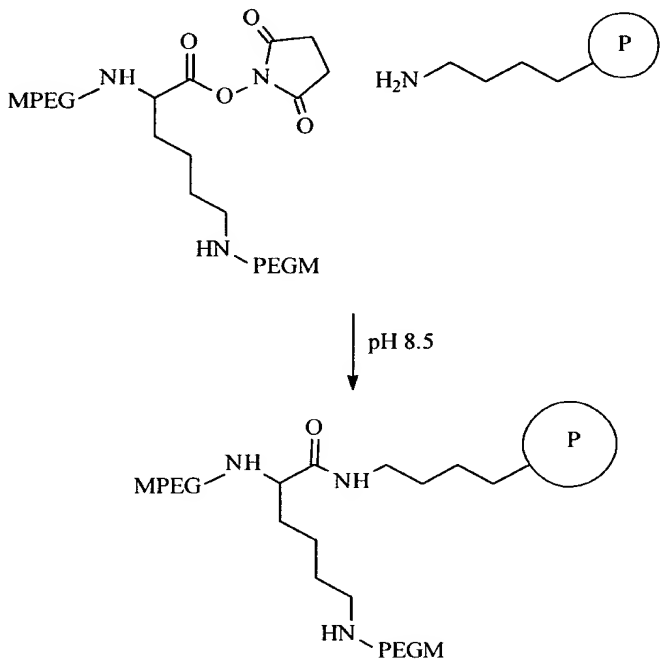


A protease moiety comprising a cysteine residue at one of the epitope protection positions is coupled with a polymer moiety according to the above scheme using the following method (wherein "P" represents the protease moiety minus the thiol group resulting from the cysteine substitution and n is the number of repeating monomer units of each polyethylene glycol (*e.g.*, n = 77)).

A variant of subtilisin BPN' with a substitution of leucine for tyrosine at position 217 and a substitution of cysteine for phenylalanine at position 17 is prepared. A concentration of approximately 2 mg / mL in phosphate buffer (pH 5.5) of the variant is achieved. The pH is then raised to 7.5 with dilute sodium hydroxide. The variant is mixed with the dimethyl polyethylene glycol maleimide at a 25:1 activated polymer to variant excess. After one hour of mixing at ambient temperature, the pH of the mixture is adjusted to 5.5 with dilute phosphoric acid and filtered through a molecular weight cut-off ultrafilter to remove excess polymer. The concentrate contains the purified protease conjugate.

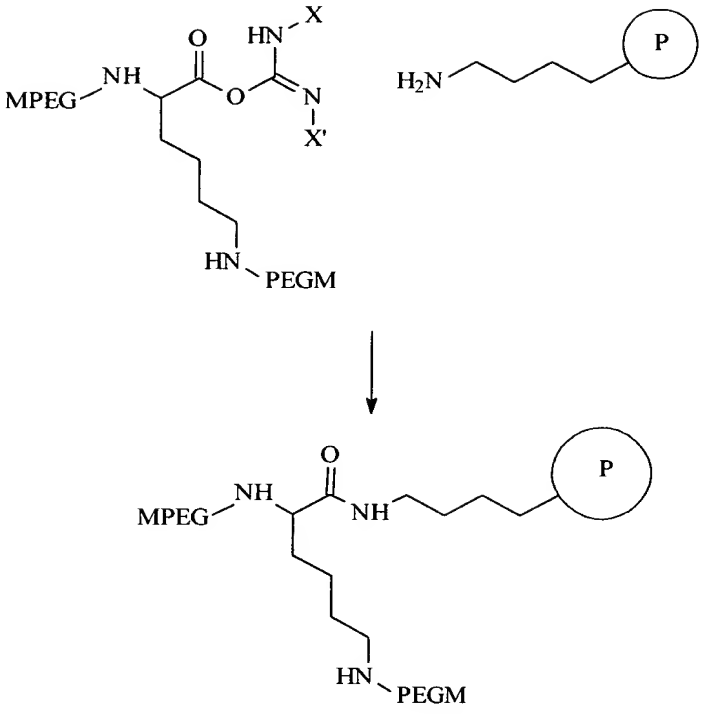
Example 3

Succinimide-protected polymer is coupled selectively to lysine in one or more of the epitope protection positions (wherein "MPEG" and "PEGM" are equivalent and represent monomethyl polyethylene glycols and wherein "P" represents the protease moiety minus the lysine amine group shown):

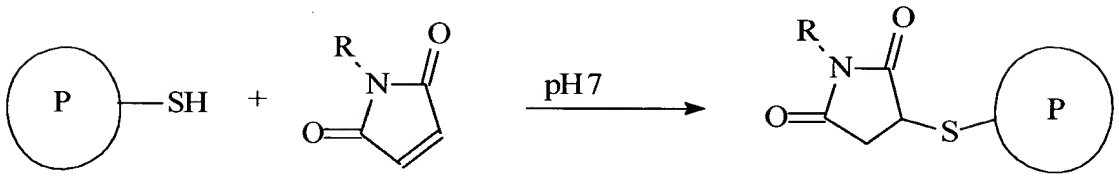


Example 4

Carbodiimide-protected polymer is coupled selectively to lysine in one or more of the epitope protection positions (wherein "MPEG" and "PEGM" are equivalent and represent monomethyl polyethylene glycols, "P" represents the protease moiety minus the lysine amine group shown, and X and X' are side chains comprising the carbodiimide moiety, for example, alkyls):



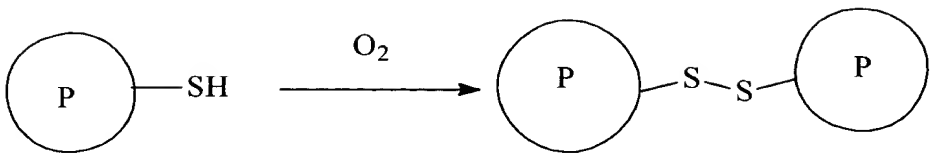
Example 5



A protease moiety comprising a cysteine residue in one of the epitope protection positions is coupled with an alkyl maleimide using the following method (wherein "P" represents the protease moiety minus the thiol group resulting from the cysteine substitution and "R" is an alkyl group). In this example, R_1 and R_2 are each nil and the linking moiety is derived from the alkyl maleimide.

A variant of subtilisin BPN' with a substitution of leucine for tyrosine at position 217 and a substitution of cysteine for serine at position 86 is prepared. A 20 mL solution of the variant is prepared at a concentration of approximately 1 mg / mL in 0.01 M KH_2PO_4 buffer (pH 7). To this solution, an 1.5 equivalents of alkyl maleimide (for example, methyl maleimide) is added to the solution. The solution is gently mixed at ambient temperature for approximately one hour. The resulting protease conjugate is obtained from the solution by standard methods.

Example 6



2 Equivalents 1 Equivalent

A protease moiety comprising a cysteine residue at one of the epitope protection positions forms a dimer using the following method (wherein "P" represents the protease moiety minus the thiol group resulting from the cysteine substitution). In this example, the protease moiety and the polypeptide moiety are equivalent (and X is nil).

A variant of subtilisin BPN' with a substitution of leucine for tyrosine at position 217 and a substitution of cysteine for serine at position 214 is prepared. A 20 mL solution of the variant is prepared at a concentration of approximately 1 mg / mL in 0.01 M KH_2PO_4 buffer (pH 8.6). Oxygen is gently bubbled through the solution at ambient temperature for approximately one hour to form the desired protease conjugate dimer. The resulting protease conjugate is obtained from the solution by standard methods.

Analytical Methods

The present protease conjugates may be tested for enzymatic activity and immunogenic response using the following methods, both of which are known to one skilled in the art. Other methods well-known in the art may alternatively be used.

Protease Conjugate Activity

The protease activity of a protease conjugate of the present invention may be assayed by methods which are well-known in the art. Two such methods are set forth herein below:

Skin Flake Activity Method

Using Scotch® #3750G tape, human skin flakes are stripped from the legs of a subject repeatedly until the tape is substantially opaque with flakes. The tape is then cut into 1 inch by 1 inch squares and set aside. In a 10 mm by 35 mm petri dish, 2 mL of 0.75 mg / mL of a control enzyme (for example, subtilisin BPN') or the protease conjugate to be tested is added in 0.01 M KH_2PO_4 pH 5.5 buffer. To this solution 1 mL of 2.5% sodium laurate pH 8.6 solution is added. The solution is gently mixed on a platform shaker. The previously prepared tape square is soaked in the solution (flake side up) for ten minutes continuing gentle mixing. The tape square is then rinsed gently in tap water for fifteen seconds. Stevenel Blue Stain (3 mL, commercially available from Sigma Chemical Co., St. Louis, MO) is pipetted into a clean petri dish. The rinsed tape

square is placed into the stain for three minutes (flake side up) with gentle mixing. The tape square is removed from the stain and rinsed consecutively in two beakers of 300 mL distilled water, for fifteen seconds per rinse. The tape square is allowed to air-dry. The color intensity between the tape square obtained from the control enzyme and the tape square obtained from the protease conjugate is compared visually or by using a chromameter. Relative to the control enzyme tape square, a protease conjugate tape square showing less color intensity is indicative of a protease conjugate having higher activity.

Dyed Collagen Activity Method

Combine 50 mL of 0.1 M tris buffer (tris-hydroxymethyl-aminomethane) containing 0.01 M $CaCl_2$ to give pH 8.6, and 0.5 g azocoll (azo dye impregnated collagen, commercially available from Sigma Chemical Co., St. Louis, MO). Incubate this mixture at 25 °C while gently mixing with a platform shaker. Filter 2 mL of the mixture through a 0.2 micron syringe filter and read absorbance of the mixture at 520 nm to zero a spectrophotometer. Add 1 ppm of a control enzyme (for example, subtilisin BPN') or the protease conjugate to be tested to the remaining 48 mL of tris / azocoll mixture. Filter 2 mL of the control / protease conjugate containing solution through a 0.2 micron syringe filter every two minutes for a total of ten minutes. For each filtered sample, read the absorbance immediately at 520 nm. Plot the results against time. The slopes of the control and the test conjugate are indicative of relative activities of the samples. A higher slope is indicative of a higher activity. The test protease conjugate activity (slope) may be expressed as a percent of the control activity (slope).

Mouse Intranasal Test for Immunogenicity

The immunogenic potential of the protease conjugates of the present invention may be determined using a methods known in the art or by the Mouse Intranasal Test for Immunogenicity presented herein below. This test is similar to the assays described in Robinson et al., "Specific Antibody Responses to Subtilisin Carlsberg (Alcalase) in Mice: Development of an Intranasal Exposure Model", *Fundamental and Applied Toxicology*, Vol. 34, pp. 15 - 24 (1996) and Robinson et al., "Use of the Mouse Intranasal Test (MINT) to Determine the Allergenic Potency of Detergent Enzymes: Comparison to the Guinea Pig Intratracheal (GPIT) Test", *Toxicological Science*, Vol. 43, pp. 39 - 46 (1998), both of which assays may be utilized in place of the test set forth herein below.

Female BDF1 mice (Charles River Laboratories, Portage, MI) weighing from about 18 to about 20 grams are utilized in the test. The mice are quarantined one week prior to dosing. The mice are housed in cages with wood chip bedding in rooms controlled for humidity (30 - 70%),

temperature (67 - 77 °F) and 12 hour light and dark cycles. The mice are fed Purina® mouse chow (Purina Mills, Richmond, IN) and water *ad libitum*.

The potential antigen to be tested (either subtilisin BPN' as positive control or a protease conjugate of the present invention) is dosed to a group of five mice. Prior to dosing, each mouse is anesthetized by an intraperitoneal (i.p.) injection of a mixture of Ketaset (88.8 mg/kg) and Rompun (6.67 mg/kg). The anesthetized animal is held in the palm of the hand, back down, and dosed intranasally with 5 µL protease in buffer solution (0.01 M KH_2PO_4, pH 5.5). While each group receives the same dosage, various dosages may be tested. Dosing solutions are gently placed on the outside of each nostril and inhaled by the mouse. Dosing is repeated on days 3, 10, 17, and 24.

Serum samples are collected on day 29. Enzyme-specific IgG1 antibody in mouse serum is measured by an antigen capture ELISA method. Immunogenicities of the protease conjugate may be compared against those of subtilisin BPN' using standard ED_{50} values.

Compositions of the Present Invention

The protease conjugates herein can be used in any application in which is suitable for the respective parent protease. One such example includes cleaning compositions. Because of the desirable reduced immunogenicity properties of the present protease conjugates, the protease conjugates may further be used in applications which have historically minimally benefited from the use of proteases. Examples of such applications include those in which the protease conjugate necessarily comes in close contact with mammalian skin (especially human skin), such as with the use of personal care compositions.

Cleaning Compositions

The protease conjugates may be utilized in cleaning compositions including, but not limited to, laundry compositions, hard surface cleansing compositions, light duty cleaning compositions including dish cleansing compositions, and automatic dishwasher detergent compositions.

The cleaning compositions herein comprise an effective amount of one or more protease conjugates of the present invention and a cleaning composition carrier.

As used herein, "effective amount of protease conjugate", or the like, refers to the quantity of protease conjugate necessary to achieve the proteolytic activity necessary in the specific cleaning composition. Such effective amounts are readily ascertained by one of ordinary skill in the art and is based on many factors, such as the particular protease conjugate used, the cleaning application, the specific composition of the cleaning composition, and whether a liquid

or dry (*e.g.*, granular, bar) composition is required, and the like. Preferably, the cleaning compositions comprise from about 0.0001% to about 10%, more preferably from about 0.001% to about 1%, and most preferably from about 0.01% to about 0.1% of one or more protease conjugates of the present invention. Several examples of various cleaning compositions wherein the protease conjugates may be employed are discussed in further detail below.

In addition to the present protease conjugates, the present cleaning compositions further comprise a cleaning composition carrier comprising one or more cleaning composition materials compatible with the protease conjugate. The term "cleaning composition material", as used herein, means any material selected for the particular type of cleaning composition desired and the form of the product (*e.g.*, liquid, granule, bar, spray, stick, paste, gel), which materials are also compatible with the protease conjugate used in the composition. The specific selection of cleaning composition materials is readily made by considering the surface material to be cleaned, the desired form of the composition for the cleaning condition during use (*e.g.*, through the wash detergent use). The term "compatible", as used herein, means the cleaning composition materials do not reduce the proteolytic activity of the protease conjugate to such an extent that the protease is not effective as desired during normal use situations. Specific cleaning composition materials are exemplified in detail hereinafter.

The protease conjugates of the present invention may be used in a variety of detergent compositions wherein high sudsing and good cleansing is desired. Thus the protease conjugates can be used with various conventional ingredients to provide fully-formulated hard-surface cleaners, dishwashing compositions, fabric laundering compositions, and the like. Such compositions can be in the form of liquids, granules, bars, and the like. Such compositions can be formulated as "concentrated" detergents which contain as much as from about 30% to about 60% by weight of surfactants.

The cleaning compositions herein may optionally, and preferably, contain various surfactants (*e.g.*, anionic, nonionic, or zwitterionic surfactants). Such surfactants are typically present at levels of from about 5% to about 35% of the compositions.

Nonlimiting examples of surfactants useful herein include the conventional C_{11}-C_{18} alkyl benzene sulfonates and primary and random alkyl sulfates, the C_{10}-C_{18} secondary (2,3) alkyl sulfates of the formulas $CH_3(CH_2)_x(CHOSO_3)^-M^+)CH_3$ and $CH_3(CH_2)_y(CHOSO_3^-M^+)$ CH_2CH_3 wherein x and (y+1) are integers of at least about 7, preferably at least about 9, and M

is a water-solubilizing cation, especially sodium, the C_{10}-C_{18} alkyl alkoxy sulfates (especially EO 1-5 ethoxy sulfates), C_{10}-C_{18} alkyl alkoxy carboxylates (especially the EO 1-5 ethoxycarboxylates), the C_{10}-C_{18} alkyl polyglycosides, and their corresponding sulfated polyglycosides, C_{12}-C_{18} α-sulfonated fatty acid esters, C_{12}-C_{18} alkyl and alkyl phenol alkoxylates (especially ethoxylates and mixed ethoxy/propoxy), C_{12}-C_{18} betaines and sulfobetaines ("sultaines"), C_{10}-C_{18} amine oxides, and the like. The alkyl alkoxy sulfates (AES) and alkyl alkoxy carboxylates (AEC) are preferred herein. The use of such surfactants in combination with the amine oxide and / or betaine or sultaine surfactants is also preferred, depending on the desires of the formulator. Other conventional useful surfactants are listed in standard texts. Particularly useful surfactants include the C_{10}-C_{18} N-methyl glucamides disclosed in U.S. Pat. No. 5, 194,639, Connor et al., issued March 16, 1993.

A wide variety of other ingredients useful in detergent cleaning compositions can be included in the compositions herein including, for example, other active ingredients, carriers, hydrotropes, processing aids, dyes or pigments, and solvents for liquid formulations. If an additional increment of sudsing is desired, suds boosters such as the C_{10}-C_{16} alkolamides can be incorporated into the compositions, typically at about 1% to about 10% levels. The C_{10}-C_{14} monoethanol and diethanol amides illustrate a typical class of such suds boosters. Use of such suds boosters with high sudsing adjunct surfactants such as the amine oxides, betaines and sultaines noted above is also advantageous. If desired, soluble magnesium salts such as $MgCl_2$, $MgSO_4$, and the like, can be added at levels of, typically, from about 0.1% to about 2%, to provide additional sudsing.

The liquid detergent compositions herein may contain water and other solvents as carriers. Low molecular weight primary or secondary alcohols exemplified by methanol, ethanol, propanol, and *iso*-propanol are suitable. Monohydric alcohols are preferred for solubilizing surfactants, but polyols such as those containing from about 2 to about 6 carbon atoms and from about 2 to about 6 hydroxy groups (*e.g.*, 1,3-propanediol, ethylene glycol, glycerine, and 1,2-propanediol) can also be used. The compositions may contain from about 5% to about 90%, typically from about 10% to about 50% of such carriers.

The detergent compositions herein will preferably be formulated such that during use in aqueous cleaning operations, the wash water will have a pH between about 6.8 and about 11. Finished products thus are typically formulated at this range. Techniques for controlling pH at

recommended usage levels include the use of, for example, buffers, alkalis, and acids. Such techniques are well known to those skilled in the art.

When formulating the hard surface cleaning compositions and fabric cleaning compositions of the present invention, the formulator may wish to employ various builders at levels from about 5% to about 50% by weight. Typical builders include the 1-10 micron zeolites, polycarboxylates such as citrate and oxydisuccinates, layered silicates, phosphates, and the like. Other conventional builders are listed in standard formularies.

Likewise, the formulator may wish to employ various additional enzymes, such as cellulases, lipases, amylases, and proteases in such compositions, typically at levels of from about 0.001% to about 1% by weight. Various detersive and fabric care enzymes are well-known in the laundry detergent art.

Various bleaching compounds, such as the percarbonates, perborates and the like, can be used in such compositions, typically at levels from about 1% to about 15% by weight. If desired, such compositions can also contain bleach activators such as tetraacetyl ethylenediamine, nonanoyloxybenzene sulfonate, and the like, which are also known in the art. Usage levels typically range from about 1% to about 10% by weight.

Soil release agents, especially of the anionic oligoester type, chelating agents, especially the aminophosphonates and ethylenediaminedisuccinates, clay soil removal agents, especially ethoxylated tetraethylene pentamine, dispersing agents, especially polyacrylates and polyasparatates, brighteners, especially anionic brighteners, suds suppressors, especially silicones and secondary alcohols, fabric softeners, especially smectite clays, and the like can all be used in such compositions at levels ranging from about 1% to about 35% by weight. Standard formularies and published patents contain multiple, detailed descriptions of such conventional materials.

Enzyme stabilizers may also be used in the cleaning compositions. Such enzyme stabilizers include propylene glycol (preferably from about 1% to about 10%), sodium formate (preferably from about 0.1% to about 1%) and calcium formate (preferably from about 0.1% to about 1%).

The present variants are useful in hard surface cleaning compositions. As used herein "hard surface cleaning composition" refers to liquid and granular detergent compositions for cleaning hard surfaces such as floors, walls, bathroom tile, and the like. Hard surface cleaning compositions of the present invention comprise an effective amount of one or more protease

conjugates of the present invention, preferably from about 0.001% to about 10%, more preferably from about 0.01% to about 5%, and more preferably still from about 0.05% to about 1% by weight of protease conjugate of the composition. In addition to comprising one or more of the protease conjugates, such hard surface cleaning compositions typically comprise a surfactant and a water-soluble sequestering builder. In certain specialized products such as spray window cleaners, however, the surfactants are sometimes not used since they may produce a filmy and / or streaky residue on the glass surface.

The surfactant component, when present, may comprise as little as 0.1% of the compositions herein, but typically the compositions will contain from about 0.25% to about 10%, more preferably from about 1% to about 5% of surfactant.

Typically the compositions will contain from about 0.5% to about 50% of a detergency builder, preferably from about 1% to about 10%.

Preferably the pH should be in the range of about 7 to 12. Conventional pH adjustment agents such as sodium hydroxide, sodium carbonate, or hydrochloric acid can be used if adjustment is necessary.

Solvents may be included in the compositions. Useful solvents include, but are not limited to, glycol ethers such as diethyleneglycol monohexyl ether, diethyleneglycol monobutyl ether, ethyleneglycol monobutyl ether, ethyleneglycol monohexyl ether, propyleneglycol monobutyl ether, dipropyleneglycol monobutyl ether, and diols such as 2,2,4-trimethyl-1,3-pentanediol and 2-ethyl-1,3-hexanediol. When used, such solvents are typically present at levels of from about 0.5% to about 15%, more preferably from about 3% to about 11%.

Additionally, highly volatile solvents such as *iso*-propanol or ethanol can be used in the present compositions to facilitate faster evaporation of the composition from surfaces when the surface is not rinsed after "full strength" application of the composition to the surface. When used, volatile solvents are typically present at levels of from about 2% to about 12% in the compositions.

Examples 7 - 12

Liquid Hard Surface Cleaning Compositions

	Ex. 7	Ex. 8	Ex. 9	Ex. 10	Ex. 11	Ex. 12
Protease Conjugate of Example 3	0.05 %	0.50 %	0.02 %	0.03 %	0.30 %	0.05 %
EDTA	-	-	2.90 %	2.90 %	-	-

Sodium Citrate	-	-	-	-	2.90 %	2.90 %
NaC_{12} Alkyl-benzene sulfonate	1.95 %	-	1.95 %	-	1.95 %	-
NaC_{12} Alkylsulfate	-	2.20 %	-	2.20 %	-	2.20 %
NaC_{12} (ethoxy) sulfate	-	2.20 %	-	2.20 %	-	2.20 %
C_{12} Dimethylamine oxide	-	0.50 %	-	0.50 %	-	0.50 %
Sodium cumene sulfonate	1.30 %	-	1.30 %	-	1.30 %	-
Hexyl Carbitol	6.30 %	6.30 %	6.30 %	6.30 %	6.30 %	6.30 %
Water	90.4 %	88.3 %	87.53 %	85.87 %	87.25 %	85.85 %

All formulas are adjusted to pH 7.

In another embodiment of the present invention, dishwashing compositions comprise one or more variants of the present invention. As used herein, "dishwashing composition" refers to all forms of compositions for cleaning dishes including, but not limited to, granular and liquid forms.

Examples 13 - 16

Liquid Dish Detergent

	Ex. 13	Ex. 14	Ex. 15	Ex. 16
Protease Conjugate of Example 1	0.05 %	0.50 %	0.02 %	0.40 %
C_{12} - C_{14} N-methyl glucamide	0.90 %	0.90 %	0.90 %	0.90 %
C_{12} ethoxy (1) sulfate	12.0 %	12.0 %	12.0 %	12.0 %
2-Methyl undecanoic acid	4.50 %	4.50 %	4.50 %	4.50 %
C_{12} ethoxy (2) carboxylate	4.50 %	4.50 %	4.50 %	4.50 %
C_{12} alcohol ethoxylate (4)	3.00 %	3.00 %	3.00 %	3.00 %
C_{12} amine oxide	3.00 %	3.00 %	3.00 %	3.00 %
Sodium cumene sulfonate	2.00 %	2.00 %	2.00 %	2.00 %
Ethanol	4.00 %	4.00 %	4.00 %	4.00 %
Mg^{2+} (as $MgCl_2$)	0.20 %	0.20 %	0.20 %	0.20 %
Ca^{2+} (as $CaCl_2$)	0.40 %	0.40 %	0.40 %	0.40 %
Water	65.45 %	65 %	65.48 %	65.1 %

All formulas are adjusted to pH 7.

Examples 17 - 19

Liquid Fabric Cleaning Compositions

	Ex. 17	Ex. 18	Ex. 19
Protease Conjugate of Example 4	0.05 %	0.03 %	0.30 %
Sodiuam C_{12} - C_{14} alkyl sulfate	20.0 %	20.0 %	20.0 %
2-Butyl octanoic acid	5.0 %	5.0 %	5.0 %
Sodium citrate	1.0 %	1.0 %	1.0 %
C_{10} Alcohol ethoxylate (3)	13.0 %	13.0 %	13.0 %
Monoethanolamine	2.50 %	2.50 %	2.50 %
Water/propylene glycol/ethanol (100:1:1)	58.45 %	58.47 %	58.20 %

Personal Care Compositions

The present protease conjugates are particularly suited for use in personal care compositions such as, for example, leave-on and rinse-off hair conditioners, shampoos, leave-on and rinse-off acne compositions, facial milks and conditioners, shower gels, soaps, foaming and non-foaming facial cleansers, cosmetics, hand, facial, and body lotions, moisturizers, patches, and masks, leave-on facial moisturizers, cosmetic and cleansing wipes, oral care compositions, catamenials, and contact lens care compositions. The present personal care compositions comprise one or more protease conjugates of the present invention and a personal care carrier.

To illustrate, the present protease conjugates are suitable for inclusion in the compositions described in the following references: U.S. Pat. No. 5,641,479, Linares et al., issued June 24, 1997 (skin cleansers); U.S. Pat. No. 5,599,549, Wivell et al., issued February 4, 1997 (skin cleansers); U.S. Pat. No. 5,585,104, Ha et al., issued December 17, 1996 (skin cleansers); U.S. Pat. No. 5,540,852, Kefauver et al., issued July 30, 1996 (skin cleansers); U.S. Pat. No. 5,510,050, Dunbar et al., issued April 23, 1996 (skin cleansers); U.S. Pat. No. 5,612,324, Guang Lin et al., issued March 18, 1997 (anti-acne preparations); U.S. Pat. No. 5,587,176, Warren et al., issued December 24, 1996 (anti-acne preparations); U.S. Pat. No. 5,549,888, Venkateswaran, issued August 27, 1996 (anti-acne preparations); U.S. Pat. No. 5,470,884, Corless et al., issued November 28, 1995 (anti-acne preparations); U.S. Pat. No. 5,650,384, Gordon et al., issued July 22, 1997 (shower gels); U.S. Pat. No. 5,607,678, Moore et al., issued March 4, 1997 (shower gels); U.S. Pat. No. 5,624,666, Coffindaffer et al., issued April 29, 1997 (hair conditioners and / or shampoos); U.S. Pat. No. 5,618,524, Bolich et al., issued April 8, 1997 (hair conditioners and / or shampoos); U.S. Pat. No. 5,612,301, Inman, issued March 18, 1997

(hair conditioners and / or shampoos); U.S. Pat. No. 5,573,709, Wells, issued November 12, 1996 (hair conditioners and / or shampoos); U.S. Pat. No. 5,482,703, Pings, issued January 9, 1996 (hair conditioners and / or shampoos); U.S. Pat. No. Re. 34,584, Grote et al., Reissued April 12, 1994 (hair conditioners and / or shampoos); U.S. Pat. No. 5,641,493, Date et al., issued June 24, 1997 (cosmetics); U.S. Pat. No. 5,605,894, Blank et al., issued February 25, 1997 (cosmetics); U.S. Pat. No. 5,585,090, Yoshioka et al., issued December 17, 1996 (cosmetics); U.S. Pat. No. 4,939,179, Cheney et al., issued July 3, 1990 (hand, face, and / or body lotions); U.S. Pat. No. 5,607,980, McAtee et al., issued March 4, 1997 (hand, face, and / or body lotions); U.S. Pat. No. 4,045,364, Richter et al., issued August 30, 1977 (cosmetic and cleansing wipes); European Patent Application, EP 0 619 074, Touchet et al., published October 12, 1994 (cosmetic and cleansing wipes); U.S. Pat. No. 4,975,217, Brown-Skrobot et al., issued December 4, 1990 (cosmetic and cleansing wipes); U.S. Pat. No. 5,096,700, Seibel, issued March 17, 1992 (oral cleaning compositions); U.S. Pat. No. 5,028,414, Sampathkumar, issued July 2, 1991 (oral cleaning compositions); U.S. Pat. No. 5,028,415, Benedict et al., issued July 2, 1991 (oral cleaning compositions); U.S. Pat. No. 5,028,415, Benedict et al., issued July 2, 1991 (oral cleaning compositions); U.S. Pat. No. 4,863,627, Davies et al., September 5, 1989 (contact lens cleaning solutions); U.S. Pat. No. Re. 32,672, Huth et al, reissued May 24, 1988 (contact lens cleaning solutions); and U.S. Pat. No. 4,609,493, Schafer, issued September 2, 1986 (contact lens cleaning solutions).

To further illustrate oral cleaning compositions of the present invention, a pharmaceutically-acceptable amount of one or more protease conjugates of the present invention is included in compositions useful for removing proteinaceous stains from teeth or dentures. As used herein, "oral cleaning compositions" refers to dentifrices, toothpastes, toothgels, toothpowders, mouthwashes, mouth sprays, mouth gels, chewing gums, lozenges, sachets, tablets, biogels, prophylaxis pastes, dental treatment solutions, and the like. Preferably, the oral cleaning compositions comprise from about 0.0001% to about 20% of one or more protease conjugates of the present invention, more preferably from about 0.001% to about 10%, more preferably still from about 0.01% to about 5%, by weight of the composition, and a pharmaceutically-acceptable carrier. As used herein, "pharmaceutically-acceptable" means that drugs, medicaments, or inert ingredients which the term describes are suitable for use in contact with the tissues of humans and lower animals without undue toxicity, incompatibility, instability, irritation, allergic response, and the like, commensurate with a reasonable benefit / risk ratio.

Typically, the pharmaceutically-acceptable oral cleaning carrier components of the oral cleaning components of the oral cleaning compositions will generally comprise from about 50% to about 99.99%, preferably from about 65% to about 99.99%, more preferably from about 65% to about 99%, by weight of the composition.

The pharmaceutically-acceptable carrier components and optional components which may be included in the oral cleaning compositions of the present invention are well known to those skilled in the art. A wide variety of composition types, carrier components and optional components useful in the oral cleaning compositions are disclosed in the references cited hereinabove.

In another embodiment of the present invention, denture cleaning compositions for cleaning dentures outside of the oral cavity comprise one or more protease conjugates of the present invention. Such denture cleaning compositions comprise an effective amount of one or more of the protease conjugates, preferably from about 0.0001% to about 50%, more preferably from about 0.001% to about 35%, more preferably still from about 0.01% to about 20%, by weight of the composition, and a denture cleansing carrier. Various denture cleansing composition formats such as effervescent tablets and the like are well known in the art (see, e.g., U.S. Pat. No. 5,055,305, Young), and are generally appropriate for incorporation of one or more of the protease conjugates for removing proteinaceous stains from dentures.

In another embodiment of the present invention, contact lens cleaning compositions comprise one or more protease conjugates of the present invention. Such contact lens cleaning compositions comprise an effective amount of one or more of the protease conjugates, preferably from about 0.01% to about 50% of one or more of the protease conjugates, more preferably from about 0.01% to about 20%, more preferably still from about 1% to about 5%, by weight of the composition, and a contact lens cleaning carrier. Various contact lens cleaning composition formats such as tablets, liquids, and the like are well known in the art and are generally appropriate for incorporation of one or more protease conjugates of the present invention for removing proteinaceous stains from contact lens.

Examples 20 - 23

Contact Lens Cleaning Solution

	Ex. 20	Ex. 21	Ex. 22	Ex. 23
Protease Conjugate of Example 5	0.01 %	0.5 %	0.1 %	2.0 %
Glucose	50.0 %	50.0 %	50.0 %	50.0 %
Nonionic surfactant (polyoxyethlene - polyoxypropylene copolymer)	2.0 %	2.0 %	2.0 %	2.0 %
Anionic surfactant (polyoxyethylene - alkylphenylether sodium sulfricester)	1.0 %	1.0 %	1.0 %	1.0 %
Sodium Chloride	1.0 %	1.0 %	1.0 %	1.0 %
Borax	0.30 %	0.30 %	0.30 %	0.30 %
Water	45.69 %	45.20 %	45.60 %	43.70 %

Examples 24 - 27

Bodywash Products

	Ex. 24	Ex. 25	Ex. 26	Ex. 27
Water	62.62 %	65.72 %	57.72 %	60.72 %
Disodium EDTA	0.2 %	0.2 %	0.2 %	0.2 %
Glycerine	3.0 %	3.0 %	3.0 %	3.0 %
Polyquaternium 10	0.4 %	0.4 %	0.4 %	0.4 %
Sodium laureth sulphate	12.0 %	12.0 %	12.0 %	12.0 %
Cocamide MEA	2.8 %	2.8 %	2.8 %	2.8 %
Sodium lauraphoacetate	6.0 %	6.0 %	6.0 %	6.0 %
Myristic Acid	1.6 %	1.6 %	1.6 %	1.6 %
Magnesium sulphate heptahydrate	0.3 %	0.3 %	0.3 %	0.3 %
Trihydroxystearin	0.5 %	0.5 %	0.5 %	0.5 %
PEG-6 caprylic / capric triglycerides	3.0 %	-	-	-
Sucrose polyesters of cottonate fatty acid	3.0 %	-	-	-
Sucrose polyesters of behenate fatty acid	3.0 %	-	4.0 %	-
Petrolatum	-	4.0 %	8.0 %	-
Mineral Oil	-	-	-	6.0 %
DMDM Hydantoin	0.08 %	0.08 %	0.08 %	0.08 %

Protease Conjugate of Example 6	0.1 %	2.0 %	2.0 %	5.0 %
Citric Acid	1.40 %	1.40 %	1.40 %	1.40 %

Examples 28 - 31

Facewash Products

	Ex. 28	Ex. 29	Ex. 30	Ex. 31
Water	66.52 %	65.17 %	68.47 %	68.72 %
Disodium EDTA	0.1 %	0.1 %	0.2 %	0.2 %
Citric Acid	-	-	1.4 %	1.4 %
Sodium Laureth-3 Sulfate	3.0 %	3.5 %	-	-
Sodium Laureth-4 Carboxylate	3.0 %	3.5 %	-	-
Laureth-12	1.0 %	1.2 %	-	-
Polyquaternium 10	-	-	0.4 %	0.4 %
Polyquaternium 25	0.3 %	0.3 %	-	-
Glycerine	3.0 %	3.0 %	3.0 %	3.0 %
Sodium Lauroamphoacetate	-	-	6.0 %	6.0 %
Lauric Acid	6.0 %	6.0 %	3.0 %	3.0 %
Myristic Acid	-	-	3.0 %	3.0 %
Magnesium sulphate heptahydrate	2.3 %	2.0 %	2.0 %	2.0 %
Triethanol amine	4.0 %	4.0 %	4.0 %	4.0 %
Trihydroxystearin	0.5 %	0.5 %	0.5 %	0.5 %
Sucrose polyesters of behenate fatty acid	2.0 %	2.0 %	-	-
Sucrose polyesters of cottonate fatty acid	3.0 %	2.0 %	-	-
PEG-6 caprylic / capric triglycerides	-	-	-	2.0 %
Petrolatum	-	-	4.0 %	-
Mineral Oil	-	-	-	2.0 %
Cocamidopropyl betaine	2.0 %	3.0 %	1.8 %	1.8 %
Lauryl dimethylamine oxide	1.0 %	1.2 %	1.2 %	1.2 %
Dex Panthenol	1.0 %	0.25 %	0.25 %	-
DMDM Hydantoin	0.08 %	0.08 %	0.08 %	0.08 %
Protease Conjugate of Example 2	1.0 %	2.0 %	0.5 %	0.5 %
Fragrance	0.2 %	0.2 %	0.2 %	0.2 %

Examples 32 - 33

Leave-on Skin Moisturizing Composition

	Ex. 32	Ex. 33
Glycerine	5.0 %	-
Stearic acid	3.0 %	-
C_{11-13} Isoparaffin	2.0 %	-
Glycol stearate	1.5 %	-
Propylene glycol	-	3.0 %
Mineral oil	1.0 %	10.0 %
Sesame oil	-	7.0 %
Petrolatum	-	1.8 %
Triethanolamine	0.7 %	-
Cetyl acetate	0.65 %	-
Glyceryl stearate	0.48 %	2.0 %
TEA stearate	-	2.5 %
Cetyl alcohol	0.47 %	-
Lanolin alcohol	-	1.8 %
DEA - cetyl phosphate	0.25 %	-
Methylparaben	0.2 %	0.2 %
Propylparaben	0.12 %	0.1 %
Carbomer 934	0.11 %	-
Disodium EDTA	0.1 %	-
Protease Conjugate of Example 4	0.1 %	0.5 %
Water	84.32 %	71.1 %

Example 34

Cleansing Wipe Composition

Propylene Glycol	1.0 %
Ammonium lauryl sulfate	0.6 %
Succinic acid	4.0 %
Sodium succinate	3.2 %
Triclosan®	0.15 %

Protease Conjugate of Example 1	0.05 %
Water	91.0 %

The above composition is impregnated onto a woven absorbent sheet comprised of cellulose and / or polyester at about 250%, by weight of the absorbent sheet.